THE DEVELOPMENT OF A UNIVERSALLY CONSERVED M2E AND HEMAGGLUTININ PEPTIDE-BASED ELISA METHOD AGAINST INFLUENZA A

PEPTIDE-BASED ELISA METHOD AGAINST INFLUENZA A

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Öz

Amaç: Influenza A'nın klinik tanısı ve kontrolünü desteklemek için spesifik tanısal ve sürveyans tekniklerinin geliştirilmesi önemlidir. Bu çalışmada, Influenza A H3 suşlarında korunmuş olan Hemagglutinin ve tüm Influenza A virüslerinde korunmuş peptid bölgesi olan M2e peptidine dayalı ELISA yöntemi geliştirilmesi amaçlanmıştır. Gereç ve Yöntem: Seçilen bu iki peptid dizisinin tanısal değeri Influenza A-hasta serum örneklerinde ELISA yöntemi ile karşılaştırılmıştır. Bulgular: M2e ve H3N2 (91-108) antijenik peptidlerine karşı antikor yanıtları incelenmiştir ve her iki peptid için tüm hastalarda cut-off değerine göre pozitif antikor yanıtları belirlenmiştir. Buna ek olarak, bu iki antijenik peptid arasında, influenza A virüsü yapısındaki işlevlerine bağlı olarak, antikor cevabı değişim gözlemlenmiştir. M2e peptidine karşı antikor cevabı, tüm H3'de suşlarında korunduğu bilinen (91-108) peptid dizisine karşı antikor cevabından daha yüksektir. Tartışma: Sonuç olarak; Influenza A pozitif hastalara ait antikor cevabı, hemagglutinin peptid sekansına ve M2e proteininin seçilen spesifik bölgesine karşı yüksek olmuştur. Influenza A virüsü düzenli olarak mutasyona girdiği için, M2e proteininin korunmuş antijenik bölgesi, Influenza A tanısında üstünlük gösterebilir.

Anahtar Kelimeler

Influenza A; Peptid; Tanı; Antijen; Antikor

Abstract

Aim: It is necessary to develop specific diagnostic and surveillance techniques to support the clinical diagnosis and control of influenza A. In this study, we aimed to develop an Enzyme Linked Immunosorbent Assay (ELISA) method based on M2e peptide sequence that is conserved in all influenza A viruses and hemagglutinin that is conserved in the H3 strain of influenza A. The diagnostic value of the two selected peptide sequences was compared by using ELISA with influenza A-patient serum samples. Material and Method: Antibody responses against the M2e and H3N2 (91–108) antigenic peptides were examined and positive antibody responses were determined according to the cutoff value for all patients in the study. In addition, variation in the antibody response was observed between these two antigenic peptides that depended on their functions in the structure of the influenza A virus. The antibody response against the M2e peptide was higher than the antibody response against the 91–108 peptide sequence known to be conserved in all H3. Discussion: In conclusion, the antibody response was high against the hemagglutinin peptide sequence and the selected specific region of the M2e protein in influenza patients. As the influenza A virus regularly mutates, the conserved antigenic region of the M2e protein sequence may show superiority in influenza A diagnosis.

Keywords

Influenza A; Peptide; Diagnosis; Antigen; Antibody.

Introduction

Influenza A viruses cause yearly epidemics of acute respiratory illness in the human population [1]. Influenza causes many deaths and hospitalizations annually. Most influenza deaths are seen in older patients (>65 years) while most influenza-related hospitalizations are with younger patients [2]. The symptoms of influenza A, i.e., cough, sore throat, myalgia, fever, and headache, [3] can also be caused by several other respiratory pathogens; therefore, laboratory diagnosis of influenza is critical for the prevention, containment, surveillance, and treatment of the associated illness [4].

Influenza can be diagnosed by viral culture, molecular tests, rapid immunochromatographic tests, and serological tests. Serological techniques still play a major role in influenza surveillance, vaccine development and evaluation, and diagnosis[5]. The Hemagglutinin Inhibition (HI) serological test is considered the "gold standard" for subtyping but this method is limited by low sensitivity and subtype cross-reactivity[6]. Influenza A viruses have structures that easily change conformation. This makes conserved and immunologically effective antigen selection extremely important for the serological diagnosis of influenza A viruses. Influenza hemagglutinin and neuraminidase glycoproteins are easily accessible on the viral membrane and highly immunogenic, but they drift and shift. However, the M2 protein (M2e) of influenza is not subject to drift or shift and has remained nearly invariable since 1918 [7].

The M2 is a transmembrane ion channel in the form of tetramers. This protein (97 amino acids) is unique to influenza type A viruses, is expressed abundantly on the surface of infected cells, and is present in small quantities on the surface of mature virions. Functioning as a pH-regulated proton channel, M2 has a role in the initial cell infection process and the formation of progeny virus [7,8]. M2e, the ectodomain of the M2-protein, is only 23 amino acids in length [7].

Studies have had different results for the antibody response against M2e of influenza A virus, with some suggesting that antibodies directed towards M2e are absent or barely detectable in human serum[9-12]. In this study, the M2e peptide conserved in all influenza A viruses and the hemagglutinin peptide sequence conserved in the H3 strain of influenza A were used for antibody detection from influenza A antibody-positive serum samples using ELISA. As such, this study developed ELISA methods based on two peptides to detect antibody response for all types of influenza A in naturally infected subjects.

Material and Method

Synthetic Peptides

Peptide sequences of influenza A virus M2e and H3N2 were commercially purchased from Bio-Synthesis Inc. (Texas, USA). The M2e peptide sequence (molecular weight (MW) 2946.28 Da and isoelectric point (pI) 6.4) was NH2-MSLLTEVETPIRNEW-GCRCNDSSD-OH. The peptide was hydrophilic and soluble in aqueous medium, so it was dissolved in water. The H3N2 (91–108) peptide (MW 2040.21 Da and pI 3.71) was NH2-SKAF-SNCYPYDVPDYASL-OH. This peptide had poor soluble property, so it was dissolved in water with stirring overnight.

Nasal Swab and Serum Samples

Ethics approval was provided by the Istanbul University, Faculty of Medicine, Clinical Research Ethics Committee. Patients with influenza-like symptoms were selected for the study. Nasopharyngeal samples were collected with Virocult swabs (Medical Wire Equipment Co., United Kingdom). The swab samples were added to 1 mL liquid medium, mixed thoroughly with a vortex mixer, and stored at 4 ºC until needed for the real-time PCR (RT-PCR). Venous blood samples were taken from the arm and centrifuged at 2000 rpm for 5 min. The serum was separated from the cells and stored at -20 ºC until needed for the ELISA.

Real-Time PCR(RT-PCR)

Viral RNA was extracted using the EZ1 Advanced XL (QIAGEN, Japan) and EZ1 Virus Mini Kit V 2.0 (QIAGEN, Germany) according to the manufacturers' instructions. Centers for Disease Control and Prevention (CDC) protocol for RT-PCR was used[13]. The primer and probe set targeting the human RNase P gene was provided by the World Health Organization as an internal positive control. RNA Virus Master (Roche, Ref No: 05619416001, Germany) enzyme mix was used for amplification in an ABI 7500 RT-PCR system (Applied Biosystems California, USA). RNA isolate (5 µL) was added to 15 µL reaction mix consisting of 8.6 µL of dH2O, 0.5 µL of primer, 1 µL of probe, 0.4 µL of enzyme, and 4 µL of reaction buffer. Amplification conditions were 50 °C for 30 min, 95 °C for 2 min, 45 cycles of 95 °C for 15 s, and 55 °C for 30 s. The amplification output of the patient serum samples was evaluated.

ELISA

Patient serum samples determined positive by RT-PCR were used for the ELISA. An indirect, noncompetitive ELISA was used, and the experiments were repeated at least 3 times with at least 5 wells used per sample. The flat bottom 96-well plate (Santa-Cruz Biotechnology, Texas, USA) was coated with 100 µL M2e peptide or H3N2 peptide at a concentration of 1 µg/mL in 50 mM carbonate-bicarbonate buffer (pH 9.6) and incubated at 4 °C overnight. The washed plates were blocked with 10 mg/mL bovine serum albumin in phosphate buffer (PBS) containing 0.05% Tween 20 (PBS-T) and incubated at 37 °C for one hour. The plates were washed with PBS-T and incubated with 1 µL of serum samples. The washing procedure was repeated, and the alkaline phosphatase conjugated anti-human IgG antibody (Millipore-AP112A, Massachusetts, USA) was added at 1:1000 dilutions. Then SIGMAFAST™ p-nitrophenyl phosphate tablets (Sigma-Aldrich, Missouri, USA) dissolved in 5 mL deionized water and 100 µL of substrate solutions were added to the wells. The optical density of the wells was read at 405 nm with a microtiter plate reader (Thermo LabSystems Multiskan Ascent 354 Microplate Photometer, USA). The cutoff value to consider specimens positive for influenza A virus was determined by calculating the mean absorbance plus two times the standard derivation (SD) from specimens determined negative for influenza viruses [14].

Results

According to the real-time PCR method, patients were categorized as either influenza positive or negative. Developed ELISA methods based on both M2e peptide and H3 peptide were used to detect specific antibody responses.

Antibody responses against the M2e and H3N2 91–108 antigenic peptides were examined and positive antibody responses were determined according to the cutoff value in all patients for both peptides. In addition, we observed a difference in the antibody response to these two antigenic peptides that depended on their functions in the structure of the influenza A virus. The antibody response against the M2e peptide, a conserved anti-
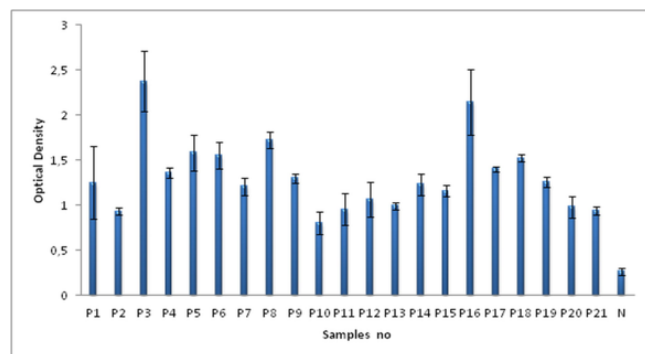

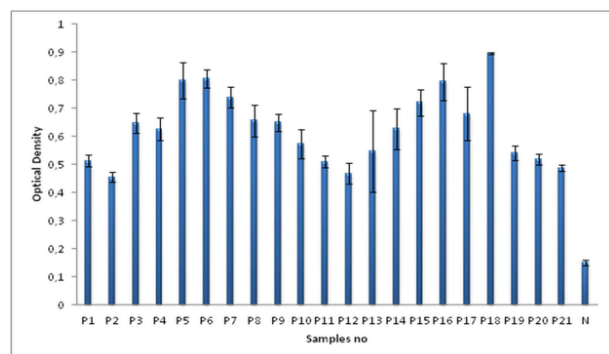Figure 1. ELISA results of patients, and negative serum samples against the M2e peptide.

Figure 2. ELISA results of patients, and negative serum samples against the H3 peptide.

genic site in the influenza A virus, was higher than the antibody response against the 91–108 peptide sequence known to be conserved in all H3.

Discussion

The M2e peptide conserved in all influenza A species and the hemagglutinin peptide sequence conserved in the H3 strain of the virus were used for antibody detection in influenza-A-positive serum samples. The hemagglutinin peptide sequence was selected because its conserved sequence is commonly used for the diagnosis of influenza A[15]. The diagnostic value of the M2e peptide is not fully known, though some results have indicated a low antibody response to whole M2e protein[16]. Therefore, the highly conserved 24-amino-acid sequence at the N-terminus domain of the M2e peptide on the outer membrane of the virus was selected to explore the antibody response against it and its diagnostic value. In this study, antibody responses against the M2e and H3 peptides were seen in acutely infected influenza A patients.

In one study, the antibody response against whole M2e protein was found negative in a 1:400 dilution ELISA for the influenza virus in both positive and negative patients[17]. In another study, an antibody response against M2e was seen in only 6 of 17 serum samples with Enzyme Immunoassay and in 12 of 17 serum samples with the more sensitive western blot assay. The studies' failure to detect anti-M2 protein antibody titers could have been due either to the sensitivity of the assay methods or to the nature of the immune response against influenza A [18]. However, these studies used whole M2e protein rather than the more conserved N-terminal 24-amino-acid domain of the protein to test for antibodies. Specific peptide-based ELISA methods have several advantages over whole protein-based methods, such as high specificity and, potentially, a simple, rapid, sensitive, and relatively inexpensive diagnostic kit[6]. In this study, positive responses against the M2e and H3 peptide sequences were seen. Accordingly, the selected peptides can be used for the diagnosis of influenza A infection not only with ELISA methods but also with other serological methods.

Competing interests

The authors declare that they have no competing interests.

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